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Evaluation of genetic diversity in fig accessions by using microsatellite markers

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ABSTRACT. Fig (*Ficus carica* L.) is a fruit of great importance worldwide. Its propagation is carried out with stem cuttings, a procedure that favors the occurrence of synonymy among specimens. Thus, molecular markers have become an important tool for studies of DNA fingerprinting, germplasm characterization, and genetic diversity evaluation in this plant species. The aim of this study was the analysis of genetic diversity among accessions of fig and the detection of synonyms among samples using molecular markers. Five microsatellite markers previously reported as polymorphic to fig were used to characterize 11 fig cultivars maintained in the germplasm bank located in Lavras, Minas Gerais. A total of 21 polymorphic DNA fragments were amplified, with an average of 4.2 alleles per locus. The average allelic diversity and polymorphic information content were 0.6300 and 0.5644, respectively, whereas the total value for the probability of identity was 1.45×10^{-4}. The study allowed the identification of 10 genotypes and 2 synonymous individuals. The principal coordinate analysis showed no defined clusters despite the formation of groups according to geographical

origin. However, neighbor-joining analysis identified the same case of synonymy detected using principal coordinate analysis. The data also indicated that the fig cultivars analyzed constitute a population of individuals with high genetic diversity and a broad range of genetic variation.

Key words: *Ficus carica* L.; Molecular markers; Homonymy; Germplasm management; Synonymy

INTRODUCTION

Fig (*Ficus carica* L.) is a traditional fruit from West Asia with a putative origin in southern Arabia or the eastern portion of Mediterranean, including Turkey and Iran, in which wild variants of this species are found (Ikegami et al., 2009). Fig is a nutritious food rich in vitamin A and minerals, and it is widely used in the food industry (Guasmi et al., 2006).

In 2010, Brazil was the 10th largest producer of fig, yielding 25.727 tons (Food and Agriculture Organization, 2012). This species has been cultivated in Brazil since the beginning of the last century, and owing to the growing demand for these fruits by the food industry, the cultivated area continues to increase each year, especially in the States of São Paulo, Rio Grande do Sul, and Minas Gerais (Paula et al., 2009).

Fig cultivation in Brazil is mainly based on a single cultivar, 'Roxo de Valinhos', which was introduced by Italian immigrants who settled in the State of São Paulo in the late 19th century (Paula et al., 2009; Francisco et al., 2011). According to Ferreira et al. (2009), the predominance of this cultivar in Brazilian orchards is due to its desired traits of vigor, productivity, rusticity, and wide acceptance by consumers and the industry. However, this predominance has resulted in serious disease risks related to nematodes (*Meloidogyne incognita* and *Heterodera fici*), fig blight (*Botrydiplodia* sp), borer (*Azochis gripusalis* Walk.), fig fly (*Zaprionus indianus* Gupta.), fig rust (*Cerotelium fici*), and mango wilt (*Ceratocystis fimbriata* Ell. & Halst.) (Rodrigues et al., 2009; Pauletti et al., 2010; Kotz et al., 2011a,b).

Fig propagation is basically carried out via the rooting of stem-cuttings (Fachinello et al., 2005), and this procedure increases the occurrence and dissemination of diseases and nematodes in orchards. Such propagation also contributes to synonymy and homonymy, because misidentification and somaclonal variations are common events in sexually propagated species such as fig (Khadari et al., 2004; Giraldo et al., 2008). The long period of domestication, the benefits of the perpetuation of beneficial traits through asexual propagation, and the exchange of materials also contribute to synonymy (Aradhya et al., 2010). Saddoud et al. (2011) reported that local fig germplasms are subject to synonymy and homonymy because the description of the genotypes is generally based on the color, format, and flavor of fruit traits frequently vulnerable to identification mistakes and environmental effects.

Various molecular markers have been used for DNA fingerprinting studies in fig as well as for germplasm characterization and analysis of genetic diversity in populations (Khadari et al., 2004; Akbulut et al., 2009; Ikegami et al., 2009; Achtak et al., 2010; Aradhya et al., 2010; Chatti et al., 2010; Dalkiliç et al., 2011). For genotype identification, molecular markers offer numerous advantages over conventional alternatives based on morphological traits because these markers are stable and detectable in all plant tissues, regardless of environmental conditions and developmental stage. The main advantages of molecular markers are the re-

duced time required for the genetic study of individuals (Agarwal et al., 2008; Gomes Filho et al., 2010) and the possibility of evaluation during seed or seedling stages.

Microsatellite markers or simple sequence repeats permit the distinction of individual heterozygotes from homozygotes, the identification of multiple alleles present in populations, and the production of easily interpretable results with high reproducibility (Alba et al., 2009). Therefore, these markers are useful and reliable tools for the genetic identification of individuals. The objective of this study was to use microsatellite markers to analyze genetic diversity and identify synonyms among fig cultivars.

MATERIAL AND METHODS

Plant material

We used 11 fig cultivars preserved in the Germplasm Bank of Empresa de Pesquisa Agropecuária de Minas Gerais (Lavras, Minas Gerais State, Brazil; Table 1). Samples of young leaves from each cultivar were obtained from a single adult plant of each accession.

Table 1. Identification of samples of fig accessions and putative geographical origin.

Identification No.	Accession	Putative origin
1	'CATI I'	Brazil
2	'CATI II'	Brazil
3	'CATI III'	Brazil
4	'CATI IV'	Brazil
5	'CATI V'	Brazil
6	'CATI VI'	Brazil
7	'Chile'	Chile
8	'Chile SN'	Chile
9	'Chile 4/5'	Chile
10	'Mission'	USA
11	'Roxo de Valinhos'	Brazil

DNA extraction

Leaf tissue was lyophilized for 48 h to reduce water content. The lyophilized tissue was then macerated in the presence of liquid nitrogen and stored in an ultra-freezer at -80°C. Genomic DNA extraction was performed according to a method described by Nunes et al. (2011). The DNA samples were visualized in the presence of ultraviolet light to check DNA quality after electrophoresis on 0.7% agarose gel stained with 0.2 µg/mL ethidium bromide and immersed in 90 mM Tris-borate, pH 8.0, and 10 mM ethylenediaminetetraacetic acid.

DNA quantification was performed by reading the absorbance at a wavelength of 260 nm using a spectrophotometer (UVmini-1240, Shimadzu, Japan). To detect contamination in the samples with protein, we also checked the absorbance at 280 nm and calculated the ratio between the 2 absorbances. Samples with a ratio of <2 were considered to be adequate.

Microsatellite marker amplification

Five microsatellite markers previously identified as polymorphic for the species *F.*

carica L. (Khadari et al., 2001) and designated MFC1, MFC2, MFC3, MFC4, and MFC7 were used in this study. The details of each marker are shown in Table 2.

Table 2. Microsatellite markers, sequences, motif, and expected sizes of alleles.

Locus identification	Primer sequences (5'-3')	Motif	Expected allele size (bp)
MFC1	F: ACTAGACTGAAAAAACATTGC R: TGAGATTGAAAGGAAACGAG	$(CT)_{13}$	192
MFC2	F: GCTTCCGATGCTGCTCTTA R: TCGGAGACTTTTGTTCAAT	$(AC)_{18}(AT)_{7}$	172
MFC3	F: GATATTTTCATGTTTAGTTTG R: GAGGATAGACCAACAACAAC	$(AC)_{15}(TC)(AC)_{8}(AT)_{7}$	136
MFC4	F: CCAAACTTTTAGATACAACTT R: TTTCTCAACATATTAACAGG	$(GA)_{13}$	218
MFC7	F: CACAATCAAAATAGTTACCG R: AGCGAAGACAGTTACAAAGC	$(AG)_{11}$	150

Amplification reactions were carried out in a final volume of 30 µL containing 50 ng DNA, 6 µL 1.5 mM 5X reaction buffer, 200 µM each of deoxyribonucleotide triphosphate, 0.5 µM of each primer (Sigma, USA), and 0.75 U Taq DNA polymerase (Go Taq Flexi, Promega, USA). The DNA amplification was developed in a gradient thermal cycler (Techne TC 5000, USA) according to Khadari et al. (2001).

To confirm the final quality of the amplification products, we poured 5 µL of each sample on 0.7% agarose gel for electrophoresis in 90 mM Tris-borate, pH 8.0, and 10 mM ethylenediaminetetraacetic acid. The gel was stained with 0.2 µg/mL ethidium bromide and visualized under ultraviolet light.

Denaturing gel electrophoresis

After confirming the amplification on agarose gels, we submitted the reaction products to electrophoresis on a 6% denaturing polyacrylamide gel under 60 W for a time determined by the expected allele size. The gel was stained with silver nitrate according to a method described by Creste et al. (2001).

Data analysis

After the allelic profiles were generated with 5 microsatellite loci, a matrix was constructed, wherein each allele from a specific locus was designated numerically from 1 to the maximum number of alleles per locus. The GenAlex 6 software (Peakall and Smouse, 2006) was used to estimate the following parameters: probability of identity (PI; for each locus and cumulative) (Waits et al., 2001), exclusion probability (for each locus and cumulative), and graphic dispersion using the methods of principal coordinate analysis (PCoA) and multilocus analysis (Excoffier et al., 2005). Powermarker version 3.25 (Liu and Muse, 2005) was used to obtain the polymorphic information content (PIC), the number of genotypes, allelic diversity (AD), and the genetic dissimilarity matrix based on the distance CS Chord (Cavalli-Sforza and Edwards, 1967). A phenetic tree was obtained using the neighbor-joining method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Allelic diversity and polymorphism detection

The microsatellite markers used were polymorphic and generated reproducible amplification fragments for all accessions evaluated. Cultivars showing only 1 allele amplified to a specific marker were considered homozygous for that locus. The 5 loci produced 21 alleles with a mean of 4.2 alleles per locus, and the number of alleles ranged from 7 in the marker MFC1 to 2 in the markers MFC2 and MFC3 (Table 3). The largest number of genotypes identified was 6 with the marker MFC7, whereas the lowest was 2 with the marker MFC2 (see Table 3). In this study, marker MFC7 displayed an efficiency higher than that reported by Chatti et al. (2010), in which this marker detected only 4 genotypes. The number of allele per loci identified in this study was also higher than that obtained by Giraldo et al. (2008) but lower than that reported by Khadari et al. (2004). These parameters indicated the existence of genetic polymorphism between the genotypes evaluated in this study.

Table 3. Genetic parameters presented by 5 microsatellite markers used in the identification and characterization of 11 fig cultivars.

Name of the marker	Number of alleles	Number of genotypes	PIC	AD	FPA	PI
MFC1	7	5	0.8142	0.8359	0.1875	0.049
MFC2	2	2	0.3398	0.4339	0.6818	0.415
MFC3	4	5	0.6132	0.6728	0.4444	0.167
MFC4	2	3	0.3698	0.4898	0.5714	0.380
MFC7	6	6	0.6848	0.7200	0.4500	0.114
Mean	4.2	4.2	0.5644	0.6300	0.4670	-
Total	21	21	-	-	-	1.45×10^{-4}

PIC = polymorphic information content; AD = allelic diversity; FPA = frequency of the principal allele; PI = probability of identity.

The average PIC value was 0.5644, whereas the maximum value was 0.8142 for marker MFC1 and the minimum value was 0.3398 for marker MFC2. Considering the PIC results obtained, 4 markers were classified as highly informative (PIC >0.5; see Table 3). Data for AD maintains a direct relationship with PIC values. Similar to the results of the PIC analysis, the highest AD was obtained for marker MFC1 (0.8359), and its value was higher than those found by Aradhya et al. (2010) and Chatti et al. (2010). In addition, the lowest AD occurred in the marker with the smallest PIC value (MFC2; 0.3398). The average AD was 0.6300 (see Table 3).

The highest frequency of the principal allele was obtained for marker MFC2 (0.6818), which also had the lowest PIC, AD, number of alleles, and number of genotypes identified (see Table 3). The lowest frequency of the principal allele was observed for marker MFC1, which also showed the highest number of alleles and highest PIC and AD values (see Table 3).

PI values for each locus ranged from 0.049 to 0.415, with the lowest for marker MFC1 and the highest for marker MFC2, respectively. Different from the lowest PIC values, the lowest PI values indicate high efficiency of the microsatellite markers applied in this study. The lowest PI values were obtained for markers MFC1, MFC3, and MFC7 (see Table 3).

The cumulative PI was 1.45×10^{-4} (Figure 1). This very low value indicated that the probability of finding 2 individuals with the same genotype profile was almost null. Working with fig genotypes, Achtak et al. (2009) observed a cumulative PI value of 2.3×10^{-4} and considered it effective for the identification of 75 accessions of the Moroccan origin.

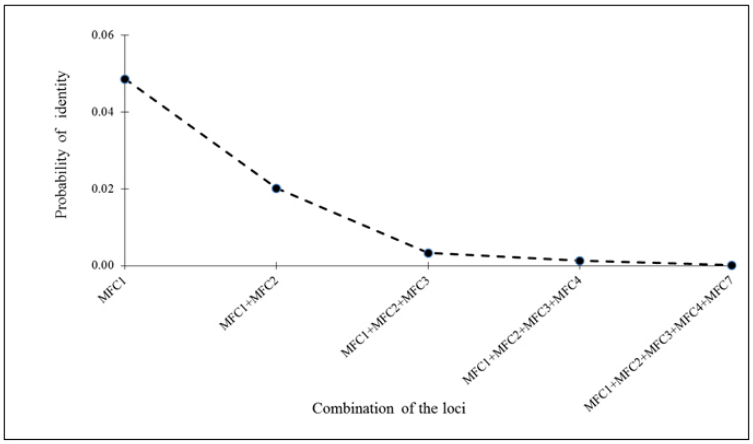

Figure 1. Variation in probability of identity (PI) according to the accumulation of the PI value of each locus.

Genetic structure

The graphical plot analysis (PCoA) showed the relationship between similarity and genetic distance among the accessions analyzed. In this study, combined coordinates explained 56.05% of the total variation, whereas the first coordinate explained 34.78% and the second coordinate explained 21.27% (Figure 2).

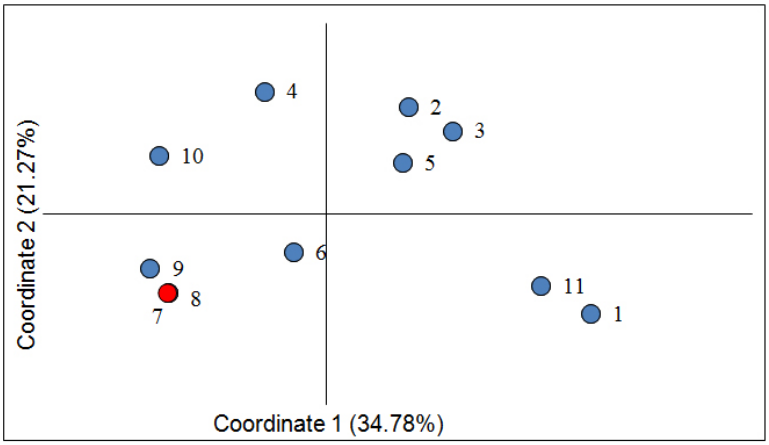

Figure 2. Results of principal coordinate analysis of 11 fig accessions. There is no genetic difference between the genotypes overlapping in the same circle (numbers 7 and 8, indicated in red).

PCoA revealed 2 synonymous accessions. Synonymy occurs when a single genotype receives 2 distinct names. In this case and according to the results of PCoA, the accessions 'Chile' and 'Chile SN' are genetically the same cultivar, although they have different denominations. In Figure

2, these cultivars are numbered 7 and 8, respectively, and are graphically positioned at the same spot.

The cultivar distribution in Figure 2 indicates high genetic diversity among the various fig accessions. The graphic distribution of accessions demonstrated that some cultivars with the same geographic region were grouped more closely than those with different geographical origins (see Table 1). Two exceptions were the cultivars 'CATI IV' and 'Mission', labeled 4 and 10, respectively, in Figure 2. The 'Mission' genotype was introduced in the United States by Spanish missionaries (Aradhya et al., 2010), whereas 'CATI IV' was introduced by the Coordenadoria de Assistência Técnica Integral in São Paulo, Brazil. Table 4 shows the results of multilocus analysis, which discriminated 10 allelic profiles from a total of 11, confirming the existence of synonymy between cultivars 'Chile' and 'Chile SN'. Cluster analysis via a neighbor-joining tree based on the dissimilarity matrix was strongly correlated with the PCoA results (Figure 3). The neighbor-joining analysis identified the same case of synonymy revealed in the cluster PCoA and multilocus analyses. The phenetic tree overlapped the same accessions, corroborating the results obtained for this study and confirming an elevated agreement among the various methods applied.

Table 4. Identification number, accessions, allelic profile, and number of cultivars with the same allelic profile.

Identification No.	Accession (cultivar)	Allelic profile	Number of cultivar/allelic profile
8	'Chile SN'	0012141233g	2
7	'Chile'	0012141233g	-
3	'CATI III'	0011120014g	1
6	'CATI VI'	1412142200g	1
4	'CATI IV'	1412222223g	1
5	'CATI V'	1612121125g	1
11	'Roxo de Valinhos'	2611001266g	1
2	'CATI II'	3511000024g	1
9	'Chile 4/5'	3512141233g	1
10	'Mission'	3512230033g	1
1	'CATI I'	6711110066g	1

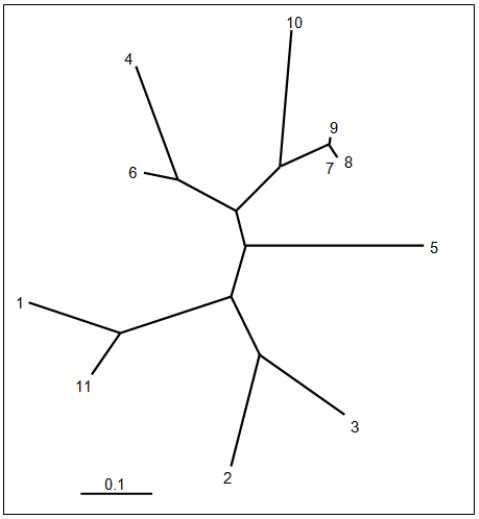

Figure 3. Phenetic tree generated by the neighbor-joining method showing the genetic relationships among 11 fig accessions. 1 = 'CATI I'; 2 = 'CATI II'; 3 = 'CATI III'; 4 = 'CATI IV'; 5 = 'CATI V'; 6 = 'CATI VI'; 7 = 'Chile'; 8 = 'Chile SN'; 9 = 'Chile 4/5'; 10 = 'Mission'; 11 = 'Roxo de Valinhos'.

The distribution of accessions in the phenetic tree demonstrated the genetic distance among these genotypes, indicating their broad genetic variation. These data are extraordinarily useful for increasing accuracy during decision making about the best parents for hybridization, thereby improving the chances of obtaining superior genotypes by crossing individuals with more highly contrasting genetic background.

CONCLUSIONS

The microsatellite markers MFC1, MFC2, MFC3, MFC4, and MFC7 provided enough genetic polymorphism among evaluated fig accessions to identify 10 distinct genotypes and 2 synonymous cultivars: 'Chile' and 'Chile SN'. The results also indicated which of the accessions analyzed are composed of genotypes with broad genetic diversity.

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